

# Metabolic and Other Responses of 'Bartlett' Pear Fruit and Suspension-cultured 'Passe Crassane' Pear Fruit Cells Held in 0.25% O<sub>2</sub>

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**Abstract.** 'Bartlett' pears (*Pyrus communis* L.) that had been stored for either 2 or 8 weeks in air at 0C were placed under an atmosphere of 0.25% O<sub>2</sub> (balance N<sub>2</sub>) at 20C for 4 days then returned to air. Control pears were kept in air at 20C. Suspension-cultured 'Passe Crassane' pear fruit cells in aging medium were treated similarly. During exposure of the fruit to 0.25% O<sub>2</sub>, loss of greenness and ethylene production were inhibited and CO<sub>2</sub> production substantially decreased. Pears that had been stored for 2 weeks at 0C ripened normally, while those that had been stored for 8 weeks at 0C failed to recover normal ethylene and CO<sub>2</sub> production upon transfer to air after a 4-day exposure to 0.25% O<sub>2</sub> at 20C. Most of the latter fruit were injured as indicated by skin browning. Acetaldehyde and ethanol content increased considerably with ripening of control fruit. Although 0.25% O<sub>2</sub>-treated fruit developed yet higher acetaldehyde and ethanol contents during treatment, the concentrations returned to or below normal during subsequent exposure to air. Pears exposed to 0.25% O<sub>2</sub> had increased pyruvate decarboxylase (PDC; EC 4.1.1.1) and alcohol dehydrogenase (ADH; EC 1.1.1.1) activities that remained high after ripening in air for 6 days. Three ADH isozymes were discernible in the 0.25% O<sub>2</sub>-treated pears, whereas only one, ADHZ, was found in control fruit. These observations imply that preclimacteric pears are both less stressed during hypoxia and have greater potential for posthypoxia repair than pears of a more advanced physiological age. Increased posthypoxia respiratory and enzymatic activity and the elaboration of new ADH isoenzymes appear to be part of the repair response. Suspension-cultured pear fruit cells responded to the atmospheric changes very much like the 8-week stored fruit and likely is a good model system to further study the effects of hypoxia on pear metabolism.

Storage in low-O<sub>2</sub> atmospheres at or near 0C is a common practice that delays chlorophyll loss, softening, and ripening of many horticultural commodities (Kader, 1986). However, for insect control, low O<sub>2</sub> treatments are more effective at higher temperatures. Even relatively short exposures of fruits to O<sub>2</sub> levels <1% at 20C or higher temperatures can induce anaerobic metabolism and cause injury (Boersig et al., 1988; Smilanick and Fouse, 1989).

Hypoxic conditions increase ethanol and acetaldehyde content in pears kept for 4 days or longer at 0 to 10C (Ke et al., 1990). These volatiles, main products of anaerobic metabolism in plants (Davies, 1980), can affect cell metabolism and cause injury (Alpi et al., 1985; Harley, 1929; Janes and Frenkel, 1978; Lidster et al., 1985). Acetaldehyde is a product of PDC activity with pyruvate as the substrate. PDC activity was found to increase under anoxia in sweetpotato roots and in some Gramineae plants (Chang et al., 1982; Kelley, 1989; Laszlo and Lawrence, 1983).

Acetaldehyde, in turn, is the substrate of ADH in ethanol synthesis. Increased transcription and translation as found for avocado (*Persea americana* Mill.) (Kanellis et al., 1991) and maize (*Zea mays* L.) (Roberts et al., 1989) ADH isozymes may be the cause of higher levels of ADH activity when anoxic conditions were present.

Hypoxic treatment of fruit and vegetables can take place immediately after harvest or after storage. Previous results have shown that pear (Boersig et al., 1988) and apple (Generozova et al., 1990) fruit stored for a few months in air at low temperatures become more susceptible to hypoxia at room temperature. To gain insight on the effects of prior storage and

physiological state on subsequent hypoxia, pears were first stored in air at 0C for 2 or 8 weeks before their exposure to 0.25 O<sub>2</sub> at 20C. The response to hypoxia was assessed by following the changes in color, respiration, ethylene production, acetaldehyde and alcohol content, PDC and ADH activity, and development of ADH isoenzymes.

Suspension-cultures of pear fruit cells have been found useful in the study of various aspects of postharvest metabolism (Romani, 1987a). Their respiration is suppressed by low (10% to 2%) O<sub>2</sub> (Brady and Romani, 1988) and rendered fermentative at O<sub>2</sub> levels <1% (Boersig et al., 1988). Hypoxic treatment and several of the analyses above were administered to cultured pear cells to discern their suitability as a model system for further study.

## Materials and Methods

*Plant material and treatments.* Mature-green 'Bartlett' pear fruit were obtained on the day of harvest from Sacramento and Lake counties, Calif. Fruit were stored at 0C until the experiments were conducted after 2 and 8 weeks of storage. All experiments with fruit were conducted at 20C.

Fruit were selected for uniformity of size and freedom from defects. Individual fruit were put in 450-ml jars and ventilated at 30 ml·min<sup>-1</sup> with air or 0.25% O<sub>2</sub> (balance N<sub>2</sub>) using a continuous flow-through system. After 4 days, the fruit were transferred to air and monitored for an additional 6 days.

Initially, and for every sampling period thereafter, three fruit were randomly chosen per treatment and each was evaluated for skin color on both cheeks, CO<sub>2</sub>, and C<sub>2</sub>H<sub>4</sub> production rates, ethanol and acetaldehyde concentrations, ADH and PDC activities, and ADH isozyme content.

Suspension-cultured 'Passe Crassane' pear fruit cells were

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Abbreviations: ADH, alcohol dehydrogenase; PDC, pyruvate decarboxylase.

grown as described by Pech and Romani (1979). The cells were held in medium lacking 2,4-D for 4 days before transfer to "aging" medium and exposure to hypoxia. Aging medium contained one-fourth the concentration of nutrients in the growth medium, no 2,4-D, but supplemented with 0.4 M mannitol and 0.015 M sucrose. Two identical experiments were conducted each with three replicate flasks per treatment. The cells were cultured and treated at 25C.

*Evaluation of quality attributes.* Maturity at the onset of each experiment was assessed on a 10-fruit sample by evaluating flesh firmness (mean of 66.8 N for the 2-week and 62.0 N for 8-week stored fruit). Flesh firmness was determined on opposite peeled cheeks with a UC Fruit Firmness Tester (Western Industrial Supply Co, San Francisco) fitted with an 8-mm plunger.

Skin color was recorded with a Gardner XL-23 Tristimulus Colorimeter (Gardner Laboratory, Bethesda, Md.) using the 'a' value, whereby a change to more positive values indicates degreening. Fruit juice was extracted with a hand-press juicer. Ethanol and acetaldehyde contents of the pear juice were measured according to Davis and Chace (1969). Frozen pear juice was thawed and a 5-ml aliquot placed in a 15-ml screw-cap test tube. The tube was sealed with a plastic septum and incubated in a water bath at 60C. After 60 min, a 1-ml-headspace sample was withdrawn with a glass syringe to determine ethanol and acetaldehyde concentrations using a HP5890A gas chromatograph (Hewlett Packard, Palo Alto, Calif.) equipped with a flame ionization detector (at 250C) and a glass column (2 mm  $\times$  1.8 m) containing 5% Carbowax 20 M on 60/80 Carbowax as stationary phase and N<sub>2</sub>, at a flow rate of 20 ml·min<sup>-1</sup>, as the mobile phase. The oven was set at 85C.

*Cell count and vitality.* The packed cell volume of the suspension-cultured cells was evaluated and used to estimate cell numbers as reported by Pech and Romani (1979). Viability of the cells was estimated by actual count after selective staining with Evans blue (0.5% w/v) (Puschmann and Romani, 1983). All data on the responses of cultured cells are calculated on a "per live cell" basis.

*Gas analysis.* The 0.25% O<sub>2</sub>, 99.75% N<sub>2</sub> mixture was regularly tested for accuracy by injecting a 10-ml gas sample into a Carle gas chromatograph (Model 111; Carle Instruments, Anaheim, Calif.) equipped with a thermal conductivity detector. Carbon dioxide production rates were evaluated using a Horiba infrared CO<sub>2</sub> gas analyzer (Model SX-2; PIR-2000R; Horiba Instruments, Irvine, Calif.). Ethylene production rates were monitored on the same replicates using a Carle gas chromatograph (Model 211) equipped with a flame ionization detector.

*Enzyme extraction.* All extraction procedures were conducted at 4C. With fruit, 5 g of tissue from the cheek area, including the skin, were homogenized in 20 ml of extraction buffer for 20 sec using a Polytron homogenizer (Brinkmann Instruments, New York). With cells, a 200-ml aliquot of suspension was taken, the cells were packed by centrifugation at 3000  $\times$  g for 3 min, ground in liquid N<sub>2</sub>, and then suspended in 20 ml of extraction buffer and homogenized. The extraction buffer contained 100 mM 2-(N-morpholino)-ethanesulfonic acid (MES) (pH 6.5) and 5 mM dithiothreitol and 0.5% soluble polyvinylpyrrolidone (PVP). The homogenate was filtered through four layers of cheesecloth and centrifuged at 27000  $\times$  g for 10 min. The supernatant was retained for the assay of ADH and PDC activities.

*Enzyme assays.* The enzyme assays were conducted immediately after extraction using the method of Chang et al (1982) with some modifications. The PDC assay mixture contained 50

mM MES (pH 6.0), 0.5 mM thiamine pyrophosphate, 5 mM MgCl<sub>2</sub>, 0.9 mM NADH, 13.5 units of ADH, 0.1-ml fruit or cell extract, and the reaction was initiated with 0.1 ml of 50 mM pyruvate (final volume 1 ml). The decline in absorbance at 340 nm (oxidation of NADH) was linear for at least 2 min, and the activity is reported as millimoles NADH oxidized per milligrams protein per minute. The ADH assay mixture contained 85 mM MES (pH 6.0), 0.9 mM NADH, 0.1 ml of extract, and the reaction was initiated with 0.05 ml of 80 mM acetaldehyde (final volume 1 ml). The decline in absorbance at 340 nm was linear for at least 3 min. Activity is reported as micromoles NADH oxidized per milligram protein per minute. Magnesium chloride concentration for PDC extraction and activity, assay pH for PDC, and extraction and assay pH for ADH are based on optima determined in preliminary experiments (data not shown).

*ADH isozyme analysis.* Pear tissue (3 g) was macerated with a Polytron homogenizer in 5 ml of extraction buffer to which 0.4 g insoluble [40,000 molecular weight (MW)] PVP had been added and then left in the refrigerator overnight as described by Arulsekhar and Parfitt (1986). The macerates were centrifuged at 3000  $\times$  g for 5 min and the supernatants stored at -80C until used. Starch gels were prepared, run, and the ADH isozymes stained according to Arulsekhar and Parfitt (1986).

*Extractable protein.* Protein content was estimated by the Bradford (1976) method using Bovine Serum Albumin (BSA) as the standard.

*Statistical analysis.* Two complete experiments were conducted on fruit from each harvest area and stored 2 weeks, i.e., a total of four experiments. Only one experiment was conducted on pears obtained from two areas and stored for 8 weeks. Two experiments were conducted with the suspension-cultured cells. Triplicate fruit or fruit cell samples per treatment were taken at each sampling period. Two-way analysis of variance over treatment and time were conducted. SE (n = 3, except for skin color, where n = 6) are given.

## Results

The response and adjustments to hypoxic stress are evident in the changes that take place during hypoxia and upon removal to air. These are documented for each of the indices measured.

*Fruit color.* Comparison of color values at the end of the storage period (day 0, Fig. 1 A and B) reveals some degreening and physiological advancement during the 8 weeks at 0C. That low O<sub>2</sub> suppressed skin color change was obvious after the first day (Fig. 1 A and B). The loss of green pigment was completely inhibited in the 2-week stored fruit (Fig. 1A) but only partially suppressed in fruit that had been stored at 0C for 8 weeks (Fig. 1B). Upon transfer to air, degreening progressed at near normal rates (Fig. 1 A and B). Prior hypoxia did not have any apparent residual effect.

*Cell viability.* The viability of cultured pear cells declined slightly (from 96% to  $\approx$  0%) during 10 days under 0.25% O<sub>2</sub> but decreased from 96% to  $\approx$  65% in a similar period in air (data not shown).

*Ethylene production.* Upon transfer to air at 20C, fruit that had been at 0C for 2 weeks exhibited a normal ethylene climacteric, reaching a peak after -5 days (Fig. 2A). Ethylene production was completely inhibited under hypoxia, but resumed upon transfer to air and exhibited a normal climacteric.

Pears that had been stored for 8 weeks at 0C exhibited a high ethylene production rate upon transfer to 20C. The pattern resembled a climacteric high followed by a postclimacteric decline

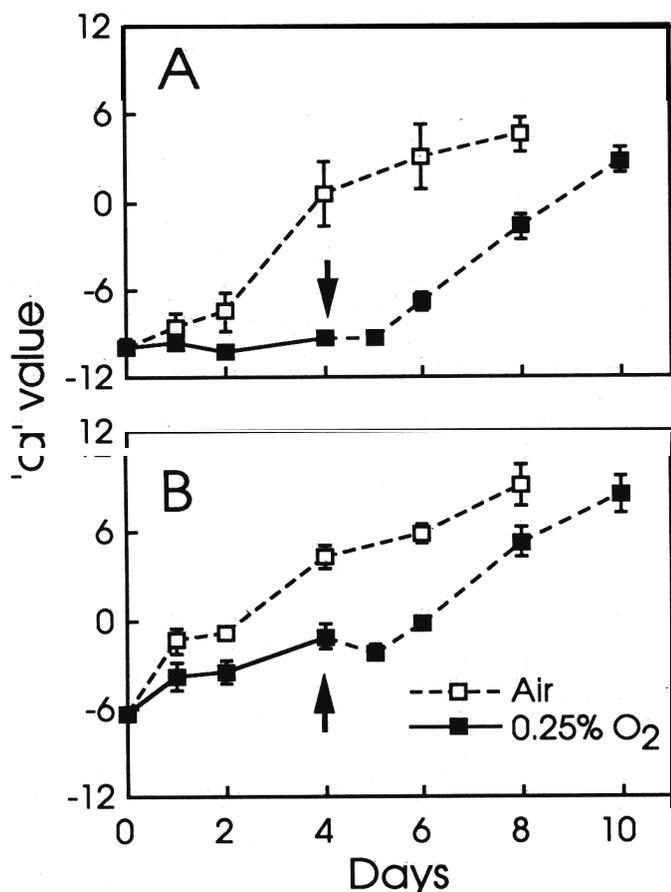


Fig. 1. Changes in the skin color of 'Bartlett' pears first stored at 0C for 2 (A) or 8 (B) weeks and then at 20C in air or in 0.25% O<sub>2</sub> with transfer to air (▼) after 4 days.

after the 2nd day (Fig. 2B). Ethylene production from pears stored for 8 weeks at 0C and then transferred to 20C under 0.25% O<sub>2</sub> was barely perceptible. Upon removal to air, the fruit exhibited only a modest and transient rise in ethylene evolution. Ethylene production by the more senescent fruit apparently was irreparably impaired by the 4-day exposure to 0.25% O<sub>2</sub>.

Ethylene production by the senescent, suspension-cultured pear cells, and the effects on it by hypoxia (Fig. 3) closely resemble the response of the 8-week pears.

**Respiration.** Changes in rates of CO<sub>2</sub> evolution by the pears followed a similar though less accentuated pattern as those for ethylene. Transfer to air at 20C after 2 weeks at 0C led to a climacteric respiratory rise that reached a peak after 6 days. An intervening 4 days of hypoxia resulted in lowered respiratory rates that recovered upon transfer to air (Fig. 4A).

Physiologic advancement of the fruit during the 8 weeks at 0C is reflected in a respiratory peak only 2 days after transfer to air at 20C, by less suppression of respiration under hypoxia, and by a slower and only partial restoration of respiratory activity upon exposure to air after hypoxia (Fig. 4B). About 90% of fruit held in 0.25% O<sub>2</sub> exhibited low O<sub>2</sub> injury, as indicated by surface browning, which increased in severity with time in air.

For the suspension-cultured cells, the suppression of respiration by hypoxia and its recovery upon transfer to air (Fig. 5) is qualitatively similar to the response of the intact fruit. The rate and magnitude of the change are more pronounced in cul-

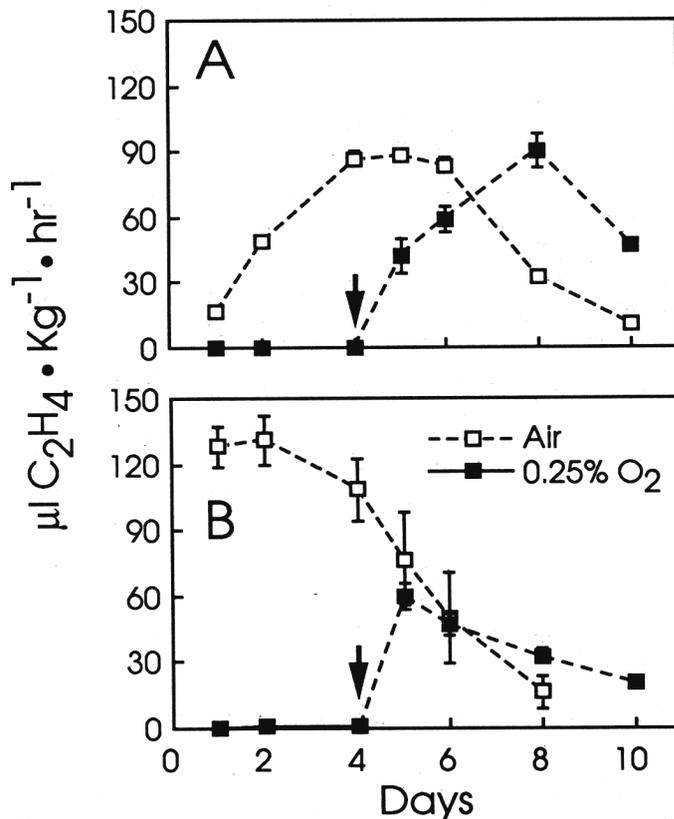


Fig. 2. Changes in ethylene production by 'Bartlett' pears first stored at 0C for 2 (A) or 8 (B) weeks and then at 20C in air or in 0.25% O<sub>2</sub> and transferred to air (▼) after 4 days.

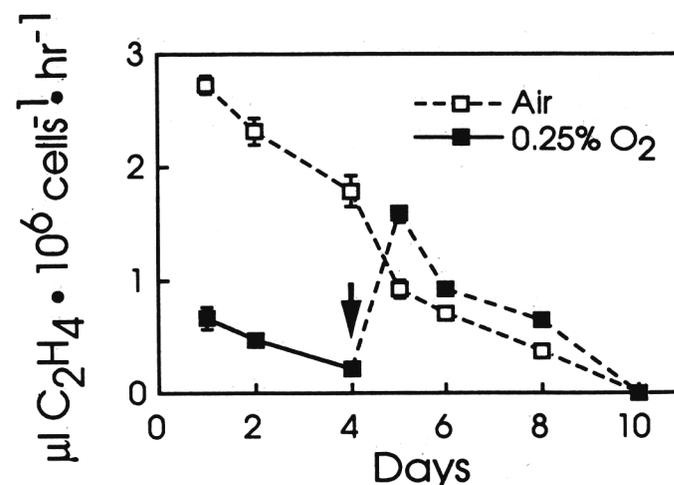


Fig. 3. Changes in ethylene production by 'Passe Crassane' pear fruit cells kept at 25C in air or in 0.25% O<sub>2</sub> and transferred to air (▼) after 4 days.

ture, likely a function of the more rapid rates of gaseous equilibrium. The rise and decline in respiration of the cells held continuously under air is not a true climacteric but rather a posttransfer metabolic adjustment and subsequent diminution of substrate (Brady and Romani, 1988).

**Acetaldehyde and alcohol content.** The acetaldehyde content of extractable juice increased several-fold as the control pear fruit ripened in air at 20C (Fig. 6A). The low O<sub>2</sub>-treated fruit

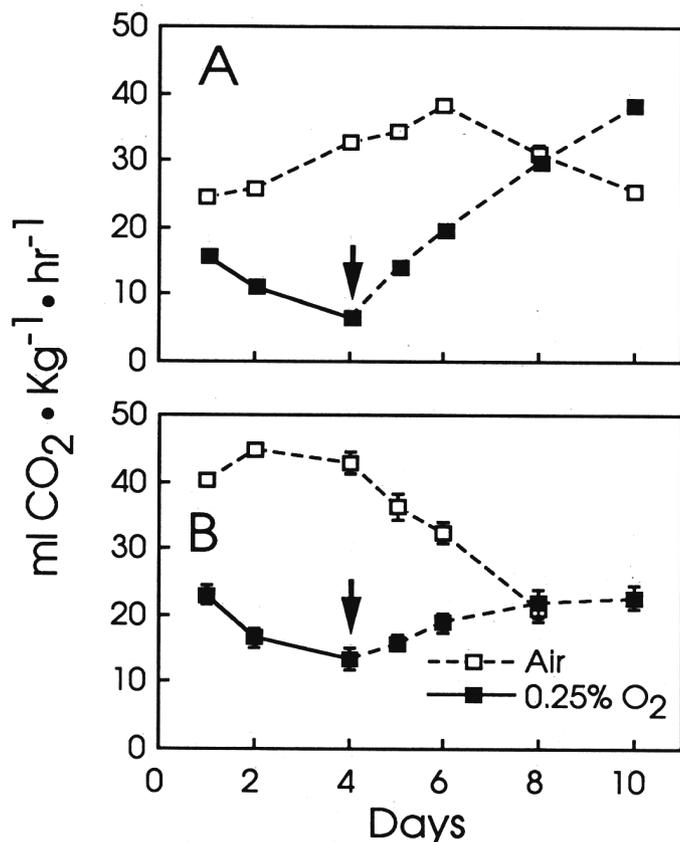


Fig. 4. Changes in  $\text{CO}_2$  production by 'Bartlett' pears first stored at OC for 2 (A) or 8 (B) weeks and then at 20°C in air or in 0.25%  $\text{O}_2$  and transferred to air (▼) after 4 days.

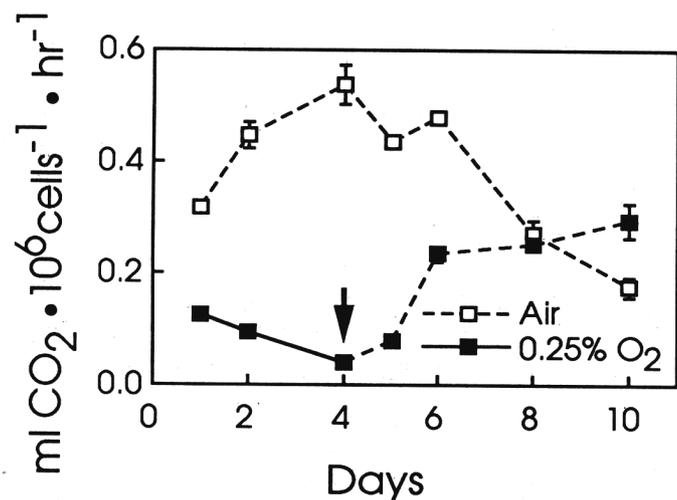


Fig. 5. Changes in  $\text{CO}_2$  production by 'Passe Crassane' pear fruit cells kept at 25°C in air or in 0.25%  $\text{O}_2$  and transferred to air (▼) after 4 days.

had a slightly higher acetaldehyde concentration during the first 2 days of hypoxia. However, after a transient rise upon transfer to air, their acetaldehyde content leveled off, resulting in a lower than normal final concentration as the fruit ripened.

Much like acetaldehyde, the ethanol content of control pear fruit increased with senescence (Fig. 6B). The ethanol content of 0.25%  $\text{O}_2$ -treated fruit reached higher levels during the treat-

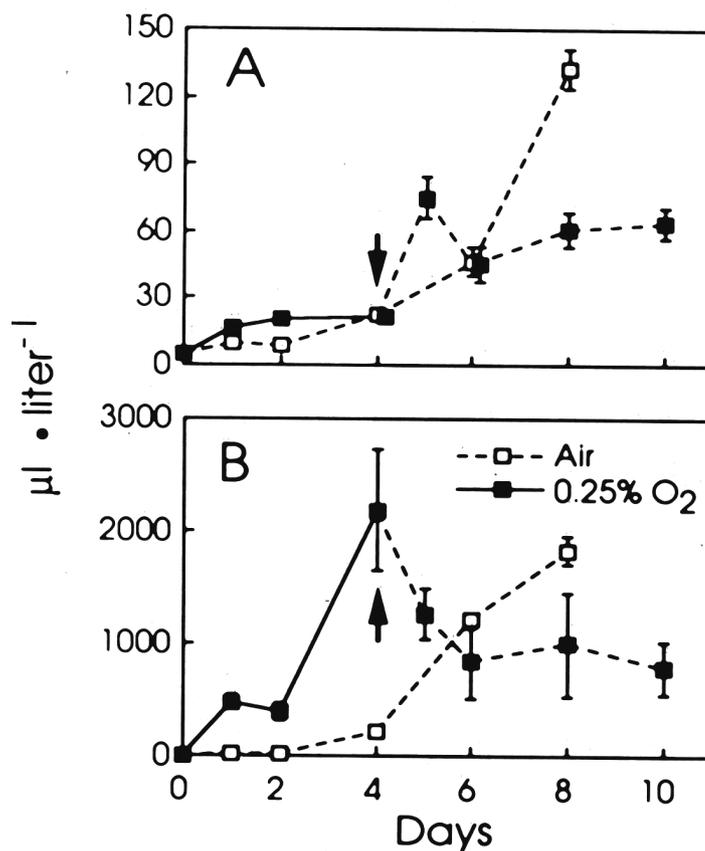


Fig. 6. Changes in the acetaldehyde (A) and ethanol (B) content of 'Bartlett' pears stored at OC for 2 weeks and then at 20°C in air or in 0.25%  $\text{O}_2$  and transferred to air (▼) after 4 days.

ment but then declined to a plateau upon transfer to air. Ethanol and acetaldehyde imply the presence of fermentation, expected during imposed hypoxia and likely furthered by poor gas exchange in the ripening tissue.

**Enzyme activity.** PDC activity in pears transferred to 20°C after 2 weeks at 0°C increased only slightly with ripening and somewhat more with an intervening 0.25%  $\text{O}_2$  treatment, but the differences were small and not always significant (data not shown). Pears transferred to 20°C after 8 weeks at 0°C also exhibited minor changes in PDC, however, those treated with 0.25%  $\text{O}_2$  had a much higher PDC activity and the activity remained high during the subsequent 6 days in air (Fig. 7). With the exception of posttransfer adjustment on the first day, the PDC activity of the cultured cells was similarly affected by hypoxia (Fig. 8).

ADH activity remained low as pears ripened in air irrespective of the length of prior cold storage (Fig. 9 A and B). However, exposure to 0.25%  $\text{O}_2$  resulted in 4- to 5-fold higher ADH activity in both short- and long-term stored fruit (Fig. 9 A and B). The activity increased with time under 0.25%  $\text{O}_2$  and remained high after transfer to air. Once again, aside from the immediate, posttransfer adjustment; ADH activity of suspension-cultured pear cells followed very much the same patterns as that of the more senescent pear fruit (Fig. 10).

**ADH isozymes.** Ripening control pear fruit had one main isozyme, ADH2, and a heterodimer band of almost similar intensity. Pears that had been exposed to hypoxia contained two additional isozymes, ADH1 and ADH3, with evidence of three heterodimers as well (Fig. 11). Although the method is mainly

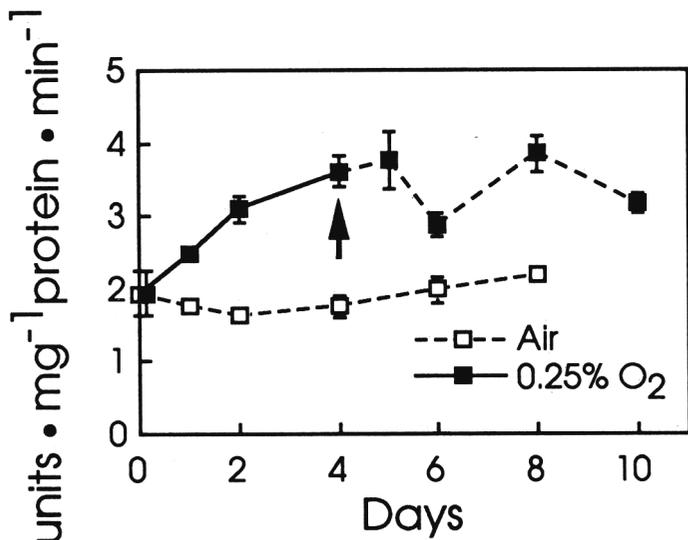


Fig. 7. Changes in the pyruvate decarboxylase activity of 'Bartlett' pears first stored at 0C for 8 weeks and then at 20C in air or in 0.25% O<sub>2</sub> with subsequent transfer to air (▼).

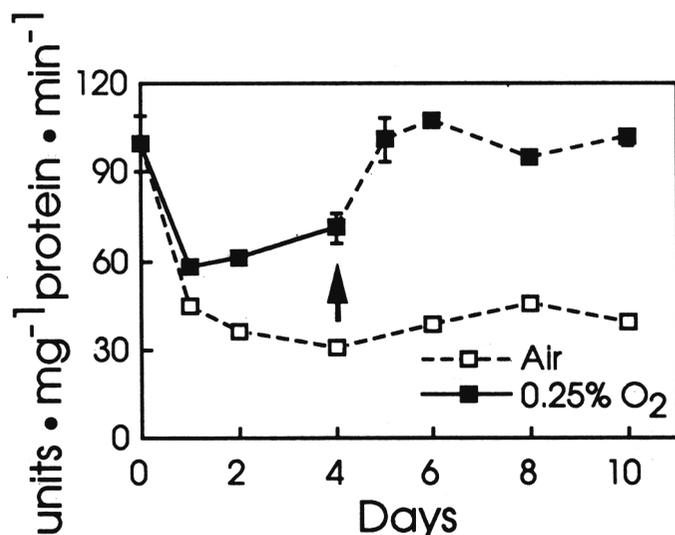


Fig. 8. Changes in the pyruvate decarboxylase activity of 'Passé Crassane' pear fruit cells kept at 25C in air or 0.25% O<sub>2</sub> with subsequent transfer to air (▼).

qualitative, an increase of ADH1 band intensity with time at low O<sub>2</sub> and its relatively stability after transfer to air is unmistakable.

### Discussion

Four days of hypoxia at 20C, a treatment that may be effective for insect control, had obvious and anticipated suppressive effects on fruit metabolism and ripening, as evidenced by little or no color change, inhibition of ethylene production, and decreased respiratory activity. That metabolism was nonetheless qualitatively affected by hypoxia was evidenced by an increase in ethanol content, a several-fold rise in PDC and ADH activity, and the elaboration of two additional ADH isoenzymes.

More indicative of fruit tolerance of hypoxia are the adjustments that take place posthypoxia and the affect thereupon of physiological state. It is clear from color readings and the ethylene and respiration data that during 8 weeks at OC the pears

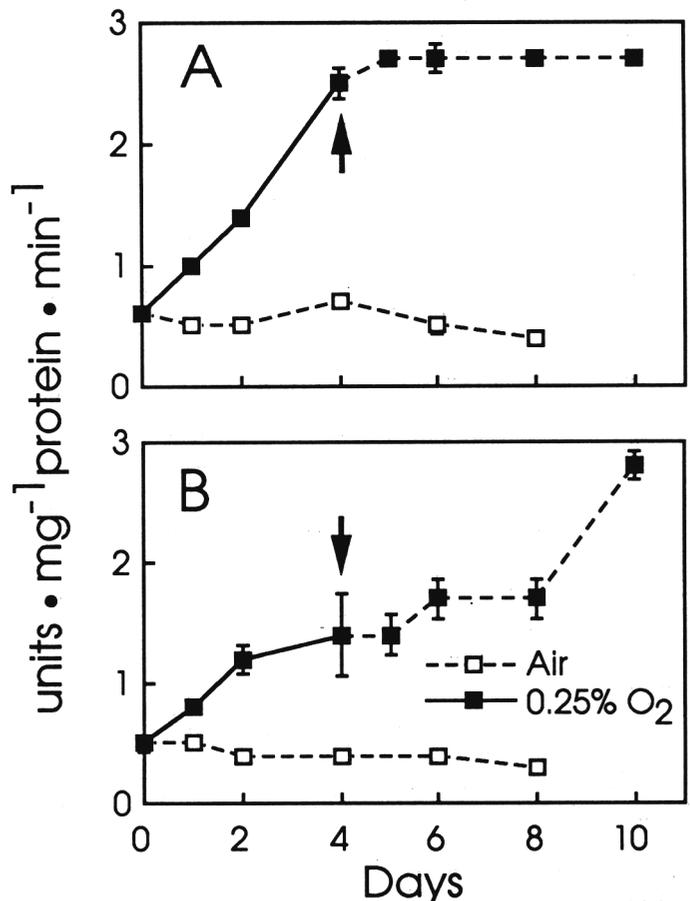


Fig. 9. Changes in the alcohol dehydrogenase activity of 'Bartlett' pears first stored at 0C for 2 (A) or 8 (B) weeks and then at 20C in air or in 0.25% O<sub>2</sub> with subsequent transfer to air (▼).

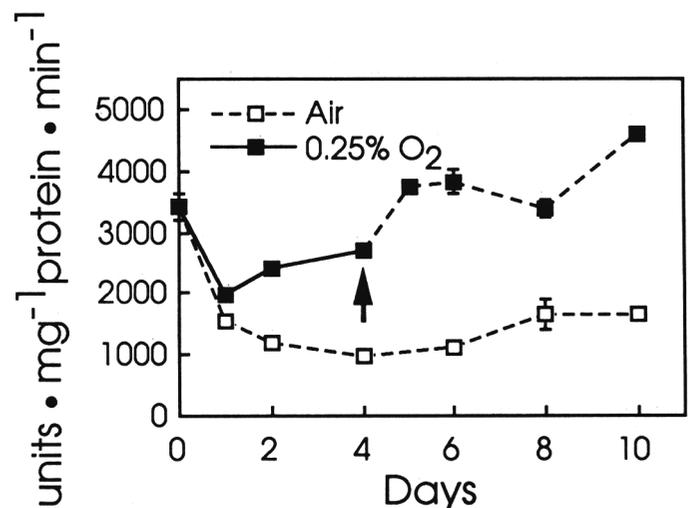


Fig. 10. Changes in the alcohol dehydrogenase activity of 'Passé Crassane' pear fruit cells kept at 25C in air or 0.25% O<sub>2</sub> with subsequent transfer to air (▼).

had advanced physiologically and were well along their respiratory climacteric rise. The more advanced physiological state appeared to have two consequences with regard to the response to hypoxia: 1) continuing fruit development, as evidenced principally by color change but also by ethylene production and respiratory rate, is not fully suppressed by hypoxia and 2) the

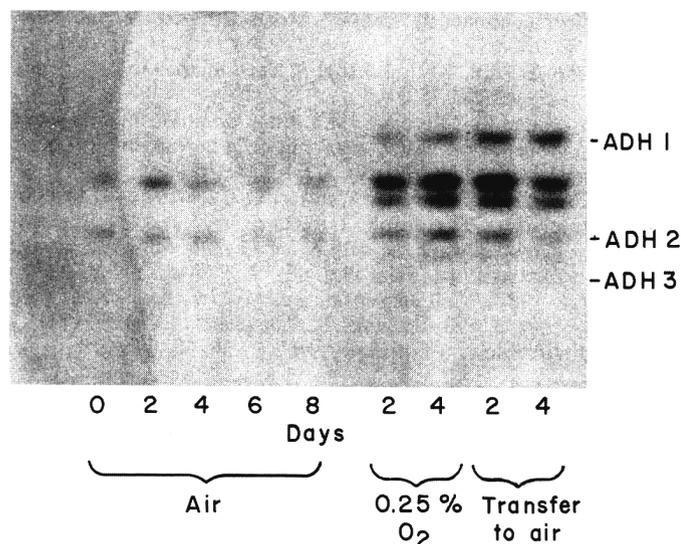


Fig. 11. Changes in alcohol dehydrogenase isozymes, ADH 1, 2, and 3, extracted from 'Bartlett' pears stored at 0C for 2 weeks and then at 20C either continuously in air or in 0.25% O<sub>2</sub> for 4 days and then transferred to air. Intergenic heterodimers are also seen exactly in the middle between each two isozymes coding for the ADH loci and are from top to bottom: ADH<sub>1</sub>/ADH<sub>2</sub>, ADH<sub>1</sub>/ADH<sub>3</sub>, and ADH<sub>2</sub>/ADH<sub>3</sub>.

posthypoxia compensatory response, as evidenced by respiration and ethylene production rates, is clearly limited. Thus, lowered tolerance to hypoxia of physiologically more mature pears may be due to both a higher metabolic activity, which increases O<sub>2</sub> demand during hypoxia, and to a decline in posthypoxia repair or homeostatic potential. The former was noted by Boersig et al. (1988) who found that ripe pears had a higher O<sub>2</sub> compensation point, evidence that fermentative respiration occurred at higher external O<sub>2</sub> levels. A decline in repair capacity during the later phases of the climacteric is predictable in the context of homeostases and senescence as discussed by Romani (1987b).

The > 4-fold increase in ADH activity during hypoxic treatment of the preclimacteric pears and its persistence after removal of the fruit to 20C controls acetaldehyde levels. Removal of acetaldehyde is physiologically important, for the compound has long been known as a cause of physiological disorder in pears (Harley, 1927, 1929) and recently was established to be much more toxic to metabolism than ethanol (Kimmerer and McDonald, 1987; Perata and Alpi, 1991). ADH activity was appreciably lower in the more senescent (8-week stored) pears (Fig. 9B), again implying a lessened compensatory or homeostatic response.

Consistent with the increase in ADH activity was the rise in ethanol content, signalling a general increase in fermentative metabolism during hypoxia. The reason for the obvious increase in the 8-week stored pears and an only minimal change in those stored 2 weeks (less senescent) is not clear. In this regard, one needs to remember that correlations between PDC and ADH activities and their product concentration are not always present, and generalizations must be made with caution (Chang et al., 1982).

Finally, in terms of several hypoxia-induced changes and subsequent response, such as suppression and recovery of ethylene production and respiration, and elevated PDC and ADC activity, the suspension-cultured pear fruit cells performed very much

like the pears that had been stored 8 weeks. The observation is consistent with pear cell behavior in response to other post-harvest related physiological/biochemical events.

*Concluding comments.* Low O<sub>2</sub> treatment did not alter the ability of short-term (2 weeks) stored pears to ripen normally as shown by color measurements and ethylene and CO<sub>2</sub> production. Their fermentation volatiles were not much higher than in ripe, control fruit and thus were not expected to influence fruit quality substantially. After a long-term (8 weeks) OC storage in air, the pears had progressed physiologically to a point near the climacteric peak and were more susceptible to hypoxic injury than those stored 2 weeks. Ethylene and CO<sub>2</sub> production rates were irreparably altered by the hypoxic treatment.

The activities of PDC and ADH increased during hypoxia, consistent with the onset of fermentative respiration but, unexpectedly; remained elevated after the fruit were transferred to air. Also, unexpected, the changes in PDC and ADH activity were essentially similar in the short- and long-term OC stored pears. Discerning the cause(s) of hypoxic injury will require, further investigation of underlying metabolic changes. In this regard, low O<sub>2</sub>-treated suspension-cultured cells behave very much like the long-term stored low O<sub>2</sub>-treated fruit in all measurements evaluated in this study. The cultured cells likely will prove a good model system to further study the effects of low O<sub>2</sub> on fruit metabolism.

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